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Antimicrobial Susceptibility of porcine *Brachyspira hyodysenteriae* isolates from Switzerland

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1 Summary

The anaerobic spirochaete *Brachyspira (B.) hyodysenteriae* is the causative agent of swine dysentery (SD), a severe mucohaemorrhagic diarrheal disease in pigs worldwide. Currently, no data for antimicrobial susceptibility of *B. hyodysenteriae* from Switzerland are available and though antimicrobial treatment is the main therapy, no standardised methods for antimicrobial susceptibility testing are established. Therefore, a broth microdilution test was performed for 30 Swiss porcine field isolates and evaluated for its suitability in diagnostics. For the pleuromutilins, tiamulin and valnemulin, a good reproducibility in testing and susceptibility at low concentrations could be observed. The reproducibility for doxycycline was acceptable. A part of the isolates showed reduced susceptibility. For lincomycin and the macrolides, tylosin and tylvalosin, reproducibility was influenced by the occurrence of so-called skips. Furthermore, reduced susceptibility for lincomycin and resistance for tylosin could be detected. Sequencing of the 23S rDNA revealed, that a point mutation is responsible for resistance and a possible explanation for the skipped wells. In a European comparison Switzerland is still in a favourable situation. However, accurate determination of minimal inhibitory concentrations is questionable, and a classification of isolates into susceptible and resistant isolates is difficult due to the lack of unambiguous classification criteria.

Zusammenfassung

Die anaerobe Spirochäte *Brachyspira (B.) hyodysenteriae* ist der ursächliche Erreger der Schweinedysenterie (SD), einer weltweit vorkommenden schweren mukohämorrhagischen Durchfallerkrankung bei Schweinen. Derzeit gibt es keine Daten aus der Schweiz zur Antibiotikaempfindlichkeit von *B. hyodysenteriae* und obwohl die Behandlung mit Antibiotika die wichtigste Therapie ist, sind keine standardisierten Methoden zur Empfindlichkeitsprüfung etabliert. Deshalb wurden 30 Schweizer Feldisolate aus dem Schwein mittels eines Mikrodilutionstests untersucht und dessen Eignung für die Diagnostik evaluiert. Für die Pleuromutiline, Tiamulin und Valnemulin, konnte eine gute Reproduzierbarkeit unter der Testung und eine Hemmung bei geringen Konzentrationen beobachtet werden. Die Reproduzierbarkeit für Doxycyclin war akzeptabel. Ein Teil der Isolate zeigte eine reduzierte Empfindlichkeit. Für Lincomycin und die Makrolide Tylosin und Tylvalosin war die Reproduzierbarkeit von sogenannten Skips beeinflusst. Darüber hinaus konnte eine reduzierte Empfindlichkeit für Lincomycin und Resistenz bei Tylosin entdeckt werden. Die Sequenzierung der 23S rDNS zeigte, dass eine Punktmutation für die Resistenz verantwortlich ist. Ausserdem stellt sie eine mögliche Erklärung für die Skips dar. Im europäischen Vergleich ist die Situation der Schweiz immer noch günstig. Trotzdem ist die akkurate Bestimmung der minimalen Hemmstoffkonzentration fragwürdig und eine Klassifizierung der Isolate in sensibel und resistent ist aufgrund des Mangels an eindeutigen Klassifizierungskriterien schwierig.

2 Introduction

2.1 Taxonomy

The order of Spirochaetales is divided into three families (*Brachyspiraceae*, *Leptospiraceae* and *Spirochaetaceae*). The family *Brachyspiraceae* includes a sole genus, *Brachyspira*. The phylogenetic analysis of *Brachyspira* is based on 16S rDNA sequencing (Paster, 2011).

Phylum: *Spirochaetes*
Class: *Spirochaetes*
Order: *Spirochaetales*
Family: *Brachyspiraceae*
Genus: *Brachyspira*

In the last decades many taxonomical reorganisations have been made and, with a large amount of non-classified species, they are still ongoing. The most relevant species in veterinary medicine is *Brachyspira (B.) hyodysenteriae*, the agent of swine dysentery. At the beginning of the 20th century, a spirochaete was described as the cause of the disease. Later, infection trials were made and the new species *Treponema hyodysenteriae* was regarded as the agent of swine dysentery (SD) (Taylor and Alexander, 1971; Glock and Harris, 1972; Harris et al., 1972). Initially, *Treponema hyodysenteriae* was grouped into pathogenic (with strong beta-haemolysis) and non-pathogenic (weakly haemolytic) strains until DNA comparison revealed a homology of only about 28% (Miao et al., 1978). Subsequently, the weakly haemolytic strains were classified in a new apathogenic species *Treponema innocens* (Kinyon and Harris, 1979). The genus name *Brachyspira* was first used for the spirochaete *Brachyspira aalborgi*, isolated from human intestine (Hovind-Hougen et al., 1982). Sequencing of the 16S rDNA in 1991 lead to the establishment of a new genus called *Serpula* including *Serpula hyodysenteriae* and *Serpula innocens* (Stanton et al., 1991). According to phylogenetic rules, the genus had to be renamed to *Serpulina* one year later since the name *Serpula* already designated a genus of fungi (Stanton, 1992). Another five years later, *Serpulina* spp. were phylogenetically compared to *Brachyspira aalborgi*, and the genera of *Brachyspira* and *Serpulina* were unified (Ochiai et al., 1997). Unfortunately, two new species, *Serpulina intermedia* and *Serpulina murdochii* were discovered at about the same time (Stanton et al., 1997) and subsequently had to be renamed to *Brachyspira intermedia* and *Brachyspira murdochii* (Hampson and La, 2006). Until now there are seven designated species of *Brachyspira*: *Brachyspira (B.) hyodysenteriae, B. pilosicoli* (Trott et al., 1996),

B. innocens (Kinyon and Harris, 1979), *B. intermedia* (Stanton et al., 1997), *B. murdochii* (Stanton et al., 1997), *B. aalborgi* (Hovind-Hougen et al., 1982), and *B. alvinipulli* (Stanton et al., 1998). Additionally, there are more species provisionally designated until today: As (potential) pathogens in pigs '*B. hampsonii'* (Chander et al., 2012; Rubin et al., 2013) and '*B. suanatina'* (Råsbäck et al., 2007), which both show a complete beta-haemolysis on blood agar, and in a diversity of other animal species and in humans '*B. christiani'* (Jensen et al., 2001), '*B. pulli'* (McLaren et al., 1997; Stephens and Hampson, 1999), '*B. canis'* (Duhamel et al., 1998), '*B. corvi'* (Jansson et al., 2008b), '*B. rattus', 'B. muris', 'B. muridarum'* (Backhans et al., 2010).

2.2 Morphology

Spirochaetes, including the genus *Brachyspira,* are characterised by their unique helical ultrastructure, which is built of three major structures: the outer sheath, periplasmic flagella and the protoplasmic cylinder. The outer sheath, the outermost layer, is trilaminar and surrounds the periplasmic space containing the endoflagella, which enables the spirochaete to be motile, and the protoplasmic cylinder (Sellwood and Bland, 1997). The flagella are connected with their proximal ends to the cylinder by insertion discs located at both poles of the cylinder. The distal ends are free and overlap with the free ends of the endoflagella from the opposite cylinder pole in the centre of the cell (Markey et al., 2013). The number of flagella varies per species and roughly equal numbers are attached to each end of the cylinder (Stanton, 2011). In contrast to most bacterial flagella, which consist of one protein (flagellin), the flagella of *B. hyodysenteriae* is composed of two sheath proteins and three core proteins (Koopman et al., 1992a). The protoplasmic cylinder, the main body of the cell, is coiled. It consists of the cell wall and the cytoplasmic membrane, which enclose the cytoplasmic contents including the nuclear material.

Brachyspira are from 2 µm to 11 µm long and from 0.2 µm to 0.4 µm in diameter (Stanton, 2011). Based on the cell size and flagellar number, species can be distinguished. *B. hyodysenteriae, B. innocens, B. intermedia, B. murdochii,* and *B. alvinipulli* are larger in cell size and have 20–30 flagella per cell. In contrast, *B. pilosicoli* and *B. aalborgi* have 8–12 flagella per cell and are shorter. Another criterion for morphological differentiation is the end shape of the cells. *B. pilosicoli* and *B. aalborgi* have pointed and tapered ends, respectively, whereas the remaining species have blunted cell ends (Stanton, 2011). Even though spirochaetes are negative in Gram staining and their outer sheath shares features

(lipooligosaccharides, which are similar to lipopolysaccharides) with the outer membrane of Gram-negative bacteria, they are phylogenetically completely distinct (Sellwood and Bland, 1997).

2.3 Metabolism

Brachyspira are anaerobes with a certain aerotolerance, and a culture atmosphere with 1% oxygen yielded higher growth rates (Stanton and Lebo, 1988). The core enzyme to reduce oxygen is a NADH oxidase (Stanton and Jensen, 1993). The NADH oxidase gene (*nox*) is present in strains throughout the genus (Stanton et al., 1995; Atyeo et al., 1999). The ability to handle oxidative stress allows *Brachyspira* to colonize the large intestine and to have a close attachment to host tissue, which represents an ecological advantage over other intestinal bacteria (Stanton, 1997). Carbohydrates are an energy source and Brachyspira ferment mono-, di- and trisaccharides as well as amino sugars (Stanton, 1997). Specific for *B. pilosicoli* is the degradation of D-ribose and the lack of beta-glycosidase (Fellström and Gunnarsson, 1995). For glycolysis, the Embden-Meyerhof-Parnas (EMP) pathway is used (Stanton, 1989). The yielded pyruvate is catabolised to acetyl-CoA, H_2 and CO_2 by the clostridial-type clastic reaction. Finally, Acetyl-CoA is degraded to acetate, butyrate or ethanol by a branched fermentation pathway. H_2 and CO_2 are produced in a 2:1 proportion (Stanton, 1989; Stanton, 1997). Additionally, a non-oxidative pentose phosphate pathway is identified (Bellgard et al., 2009). *B. murdochii* and *B. pilosicoli* encode genes for enzymes of the incomplete tricarboxylic acid cycle, which are missing in *B. hyodysenteriae*. Besides, genes of the glycine reductase complex are found in *B. murdochii* and *B. pilosicoli* (Wanchantuek et al., 2010).

Cholesterol (Lemcke and Burrows, 1980) and phospholipids (Stanton, 1987) are essential for the growth of *B. hyodysenteriae*. Cholesterol, for the most part converted to cholestenol, is used for membrane synthesis (Stanton, 1987). The capacity for fatty acid and lipid biosynthesis exists and beta-oxidation of long-chain fatty acids is possible (Bellgard et al., 2009). The complete set of genes encoding nucleotide metabolism and lipooligosaccharide biosynthesis are present (Bellgard et al., 2009).

B. hyodysenteriae expresses iron-regulated proteins, and a periplasmic iron import system has been described (Li et al., 1995; Dugourd et al., 1999). For the iron acquisition in *B. pilosicoli* the gene for a ferrous transporter has been found (Mappley et al., 2012).

2.4 Genomics

Until now the complete genomes of *B. hyodysenteriae*, *B. pilosicoli*, *B. murdochii* and *B. intermedia* have been sequenced (Bellgard et al., 2009; Pati et al., 2010; Wachanthuek et al., 2010; Håfström et al., 2011; Mappley et al., 2012; Lin et al., 2013). Comparative studies of protein-coding sequences reveal more matches with other intestinal bacteria like *Clostridium* and *Escherichia* than with other spirochaetes, reflecting the adaption to their common habitat (Bellgard et al., 2009).

In general, Brachyspira have a singular circular chromosome with an average G+C content of about 26%. The genome size differs per species between 2.4 and 3.2 Mbp (Stanton, 1997). More specialised species like *B. hyodysenteriae* and *B. pilosicoli* underwent a reductive genome evolution. Interestingly, different *B. pilosicoli* strains vary in genome size (Mappley et al., 2012). Genome comparison reveals that the swine pathogens *B. hyodysenteriae* and *B. pilosicoli* share the least number of genes and differ in their genetic organisation (Zuerner et al., 2004). They went through different phylogenetic developments in which *B. pilosicoli* separated earlier and is considered the older pathogen (Håfström et al., 2011; Mappley et al., 2012). Furthermore, a plasmid has been found in *B. hyodysenteriae, B. intermedia* and *B. murdochii* isolates but not yet in *B. pilosicoli* (Bellgard et al., 2009; Wachanthuek et al., 2010; Håfström et al., 2011; Mappley et al., 2012). A bacteriophage was detected and later identified and classified as the general transducing bacteriophage VSH-1 in *B. hyodysenteriae* and *B. innocens* (Humphrey et al., 1995; Humphrey et al., 1997). It is the only known natural gene transfer mechanism of *B. hyodysenteriae* (Stanton, 1997). VSH-1 is inducible by mitomycin C, carbadox and metronidazole, and a transmission of chloramphenicol and tylosin resistance could be proved *in vitro* (Stanton et al., 2008). Genomic studies give hints for the existence of bacteriophages in *B. intermedia*, *B. pilosicoli* and *B. murdochii,* and bacteriophages that are inducible by mitomycin C could be detected in cultures of *B. pilosicoli* (Calderaro et al., 1998a/b; Stanton et al., 2003). Due to genetic transfer mechanisms, the genetic population structure of *B. hyodysenteriae* and *B. pilosicoli* is epidemic (Trott et al., 1998). That means the population appears clonal because of certain clonal groups being dominant although recombination occurs (Maynard Smith et al., 1993). In general, Brachyspira are genetically diverse, and gene transfer not only occurs within a species but also between different species (Humphrey et al., 1995).

2.5 Pathogenicity

The intestinal spirochaete with the greatest veterinary significance is *B. hyodysenteriae* as the agent of SD in pigs. The disease is characterised by mucohaemorrhagic diarrhoea. It is one of the most relevant porcine intestinal infections worldwide. Prevalences in Europe differ from 2.5% to 14% in Denmark, 13% in the United Kingdom, 10.81% in Italy, and 32.1% in Spain (Møller et al., 1998; Stege et al., 2000; Thomson et al., 2001; Merialdi et al., 2003; Carvajal et al., 2006). In a Swedish study, *B. hyodysenteriae* could not be detected in the investigated herds even though the disease occurs in the country (Jacobson et al., 2005). In general, *B. hyodysenteriae* is isolated from a broad host spectrum including wildlife and domestic animals. Some animal species such as rheas show typhlocolitis with pathological lesions which are similar to those found in infected pigs (Sargatz et al., 1992; Jensen et al., 1996). Gross lesions were also found in wild mice but pathogenicity remains unclear (Joens and Kinyon, 1982). The focus of interest rather lies on the potential of wildlife being a reservoir for SD than on the infection in the animals. Especially wild mice and rats (Joens and Kinyon, 1982; Backhans et al., 2011) and free-living birds (Jansson et al., 2004) are considered to play a role in transmission of *B. hyodysenteriae* between farms as well as in its spreading and persisting within a farm. Furthermore, *B. hyodysenteriae* could be isolated from wild boar in Australia, whereas no *Brachyspira* spp. could be detected in Sweden (Fellström et al., 2002; Jacobsen et al., 2005; Phillips et al., 2009). Additionally, insects and indirect transmission may have a greater impact on the spreading of disease than originally thought (Blunt and McOrist, 2008; Blunt et al., 2010; Alvarez-Ordóñez et al., 2013). *B. hyodysenteriae* is known to survive in faeces up to 112 days at a temperature of 10°C (Boye et al., 2001).

Within a herd, the agent is transmitted faecally-orally. The outcome of disease is influenced by age, stress, production of acid in the stomach, nutrition, infectious dose and virulence (Alvarez-Ordóñez et al., 2013). Incubation time is about 14 days. The course varies from inapparent or mild to severe with high mortality especially in native herds. In classical cases, animals show a slight depression with reduced, but preserved, desire for food. Faeces are watery, porridge-like and dark-coloured. With the progression of disease, it contains mucus and fresh blood. A typical sign seen in milder courses are tucked-in flanks. Mortality is due to reduced food and water partake and a following dehydration. Most animals gradually recover within two weeks. Feed conversion often is reduced for the remaining period of production. Mainly concerned are weaners and growers in fattening farms. Studies including animals up to the finishing period show higher prevalences for SD. The onset of disease might be

strongly influenced by the preventive use of antimicrobials and can first occur in the finishing period when all medications are removed to avoid antimicrobial residues in meat (Hampson et al., 1997). Therefore, an increase of postweaning diarrhoea was reported after the ban of growth promoters in Sweden and Europe (Robertsson and Lundeheim, 1994; Stein, 2002). Nevertheless, in a Danish study, *B. hyodysenteriae* could be isolated in equal numbers irrespectively of the use of food additives in the farms (Møller et al., 1998). The missing of typical symptoms of SD in breeding farms is due to the endemic spread with accompanied immunity in sows or, especially in Switzerland, due to the high-health status of breeding herds. In a study from Spain, *B. hyodysenteriae* could be isolated from swine of all ages including sows, and all of them showed symptoms of diarrhoea (Carvajal et al., 2006).

SD is a multifactorial disease, and due to the complexity of different factors, pathogenesis is not fully understood (Hampson et al., 2006). Diet, housing and exposure to stress have a significant impact on occurrence and course of disease (Jacobsen et al., 2005). Besides, a synergistic interaction with other anaerobic bacteria of the intestinal microbiota facilitates colonization and development of lesions (Meyer et al., 1974; Whipp et al., 1979). Undoubtedly, there is an influence of feeding to expression of SD. Nevertheless, it is still controversially discussed because preventive effects of diets are often not reproducible, which underlines the multifactorial character of the infection. A diet composed of cooked rice and animal protein showed a protective effect (Siba et al., 1996), which was explained with a reduced fermentation in the large intestine because of lesser amounts of resistant starch and soluble non-starch polysaccharides in the diet (Pluske et al., 1996; Pluske et al., 1998; Durmic et al., 2000; Durmic et al., 2002). Nevertheless, these findings could not be reproduced (Kirkwood et al., 2000) and contradict a recent studies according to which a diet with fermentable carbohydrates deriving from dried chicory roots and sweet lupines also protected animals from SD (Thomsen et al., 2007). High concentration of inulin in this diet could be responsible for this effect (Hansen et al., 2010; Hansen et al., 2011). Additionally, numbers of *Bifidobacterium thermacidophilum* and *Megasphaera elsdenii* were increased in the microbiota of animals fed with chicory roots and sweet lupines (Mølbak et al., 2007), but previously described changes in the gut flora (Leser et al., 2000) as a consequence of an infection with *B. hyodysenteriae* could not be observed. An indirect influence of diet due to changes in the composition of the intestinal microbial flora should be kept in mind, since *in vitro B. hyodysenteriae* could be inhibited by *Lactobacillus* spp., *Bifidobacterium thermophilum*, *Enterococcus faecium* and *Bacillus subtilis* (Bernardeau et al., 2009; Klose et al., 2009).

Furthermore, clinical outcome may be influenced by variations in virulence among *B. hyodysenteriae* strains (Kinyon et al., 1977; Hyatt et al., 1994; Thomson et al., 2001; La et al., 2011). However, the occurrence of avirulent strains must be taken with caution since taxonomical classifications are not completed and avirulent strains sometimes show atypical phenotypical and genetic features (Thomson et al., 2001). Great progress in virulence determination was achieved when genetic manipulation became possible. Cloning of haemolysin and flagellar genes of *B. hyodysenteriae* (Muir et al., 1992; Koopman et al., 1992b) and gene transfer through electroporation and homologous recombination (ter Huurne et al., 1992) were the first successful attempts. Prior to that, biochemical investigations, *in vitro* assay with lysates as well as comparison of *B. hyodysenteriae* with apathogenic species were used for the description of virulence determinants. For a long time, the main criterion for distinguishing *B. hyodysenteriae* from apathogenic species was the strong beta-haemolysis. Therefore, haemolysins are considered to be one of the main virulence factors. The haemolysin is an oxygen-resistant, heat-labile protein with no lipolytic or proteolytic activity, and it is active within a broad range of pH values (Saheb et al., 1980a). Reported size varies from 19 kDa (Kent et al., 1988) to 74 kDa (Saheb et al., 1980a). Haemolysis activity is temperature-dependant (Saheb and Lafleure, 1980b). A cytotoxic effect on several eukaryotic cell lines (Muir et al., 1992) is observed, and enterocytes exposed to purified haemolysin developed lesions similar to those seen in SD (Lysons et al., 1992). Inactivation of a haemolysin gene by homologous recombination produced still haemolytic but less virulent mutants (ter Huurne et al., 1992). In further investigations three genes (*tyl*A, *tyl*B, *tyl*C) were determined to be responsible for haemolysis (ter Huurne et al., 1994). *Tyl*B- and *tyl*C-related genes are present in *B. innocens* (ter Huurne et al., 1994). However, protein sequences of purified beta-haemolysin did not correlate with the cloned gene sequences. Subsequently, a different haemolysin gene (*hyl*A) was detected, cloned and expressed in *Escherichia coli* (Hsu et al., 2001). Hybridisation indicates that *hyl*A is present in *B. hyodysenteriae* and *B. intermedia* but not in *B. innocens*. Regarding the virulence, *tylA* mutants showed a reduced virulence in pigs and had the potential to protect pigs against challenge with wild-type strains (Hyatt et al., 1994). The responsible immune response for the protection is yet unclear. Serum of convalescent pigs did not react in Western blot with recombinant haemolysin (Muir et al., 1992). Even if animals infected with the mutant strain did not develop clinical signs, the shedding of *B. hyodysenteriae* was observed indicating that colonisation by the mutant strain is still possible (Hyatt et al., 1994). Nevertheless, in the same study different wild-type strains distinguished in their virulence regardless their haemolytic activity. Therefore, haemolysin

may be only one factor contributing to pathogenesis. Stanton and Cornell (1987) suggest erythrocytes as source for essential lipids for growth. Haemolysis of red blood cells may facilitate the supply of *B. hyodysenteriae* with nutrients. Additional important factors for the colonisation of the large intestine are chemotaxis and motility. A close association of *B. hyodysenteriae* with colonic mucosa is observed in electronic microscopy, while attachment and penetration or invasion of intact epithelium was not detected (Kennedy et al., 1988). Mainly, *B. hyodysenteriae* was present in the mucus-filled crypts of Lieberkühn and the mucus gel covering the epithelium. Furthermore, *B. hyodysenteriae* was highly motile in intestinal mucus and chemotactic to porcine mucus and hog gastric mucus (Kennedy et al., 1988; Milner and Sellwood, 1994). An attempt including different *Brachyspira* strains demonstrated that virulent strains of *B. hyodysenteriae* were more chemotactic than avirulent *B. hyodysenteriae* strains, *B. pilosicoli*, *B. intermedia* and *B. innocens* (Milner and Sellwood, 1994). Genetic disruptions of flagellar genes (*fla*A1, *fla*A2, *fla*B1, *fla*B2, *fla*B3) led to reduced motility and velocity (Rosey et al., 1995; Rosey et al., 1996; Li et al., 2000) and reduced virulence and colonisation in mice (Rosey et al., 1995; Rosey et al., 1996). Similar results could be detected for *nox*-deleted mutants in swine. Fewer animals were colonised by the mutants and symptoms were less severe compared to the wild-type strain (Stanton et al., 1999). A well characterized virulence factor in Gram-negative bacteria are lipopolysaccharides (LPS). LPS are components of the cell wall of Gram-negative bacteria, which are antigenic and play a major role as endotoxins in the pathogenesis of bacterial infections. Their release of bacterial cells activates the immune system. Cytokines such as tumour necrosis factor (TNF) and Interleukin-1 (IL-1) are produced, and inflammation up to septic shock can occur (Valentin-Weigand, 2011). In the case of *B. hyodysenteriae*, a specific antigen could be extracted by hot phenol-water extraction which was assumed to be an LPS (Baum and Joens, 1979) until the determination of the molecular weight revealed it to be a lipooligosaccharide (LOS) (Halter and Joens, 1988). *In vitro*, LOS of *B. hyodysenteriae* was toxic for murine peritoneal macrophages, increased the internalisation of erythrocytes mediated by complement and immunoglobulin G-Fc receptors, enhanced the migration of porcine leucocytes and stimulated proliferation of mouse splenocytes (Nuessen et al., 1982). Endotoxin preparations (butanol/water extraction) showed a stronger biological activity and induced IL-1, IL-6 and TNF production in mice and swine (Greer and Wannemuehler, 1989b; Nibbelink et al., 1997). However, the comparison of the biological activity of LOS and endotoxin extracted from *B. hyodysenteriae* and the apathogenic *B. innocens* did not provide indications for the different pathogenic potential (Greer and Wannemuehler, 1989a).

The influence of the host was studied with C3H/HeJ mouse strains, which are hyporesponsive to LPS and did not develop gross lesions in the intestine after experimental infection (Nuessen et al., 1983). Additionally, the injection of extracted LOS had no lethal effect. Gross lesions and lethality were seen in LPS-susceptible mouse strains. However, macrophages isolated from the C3H/HeJ mice were chemotactic to LOS (Nuessen et al., 1983). These findings could be confirmed by Nibbelink and Wannemuehler (1991), who observed different susceptibility to infection depending on the mouse strain. Therefore, host responsiveness to LPS may contribute to the pathogenesis. Broad access to molecular techniques opened new possibilities for virulence determination. Due to whole genome sequencing, a plasmid could be detected in *B. hyodysenteriae* WA1 (Bellgard et al., 2009) containing potential virulence genes. The plasmid was missing in a known avirulent strain and, after an investigation of 264 Australian field isolates, another isolate without plasmid was detected and examined for virulence in experimental infection of pigs (La et al., 2011). Since it was less virulent than the control strain, the genes located on the plasmid may be involved in pathogenesis of SD. An examination of a German collection of *Brachyspira* spp. for different virulence-associated genes revealed high genetic heterogeneity, and no uniform pattern among *B. hyodysenteriae* could be detected (Barth et al., 2012). Conclusively, pathogenesis and virulence of *B. hyodysenteriae* are still not fully understood. All of the described factors reflect the adaptation of *B. hyodysenteriae* to its habitat and confirm the close association of virulence and lifestyle of the organism (Wassenaar and Gaastra, 2001).

2.6 Diagnosis

Due to the variation in the course of disease, only a presumptive diagnosis can be made based on clinical signs. Diarrhoea with blood and/or mucus is the most indicative symptom, but dehydration, depression and increase of temperature are observed as well. Haematological changes include increase of leucocytes, erythrocyte sedimentation and fibrinogen levels, left shift of neutrophils and significant electrolyte imbalances (Waldmann and Lindemann, 1990; Hampson et al., 2006). Besides, clinical handling includes farm history, evaluation of production form, biosecurity measurements and performance data. The immunity seen in pigs which recovered from infection prompted investigations of specific antibodies against *B. hyodysenteriae* and efforts for the development of serological tests. However, the diagnostic value of serological tests remains questionable, since serogroup specificity, in the case of LOS, and cross-reactivity between *Brachyspira* spp. are observed (Halter and Joens,

1988; Kent et al., 1989). Up to now, the only potential application of a serological test is the use of an indirect Enzyme-linked Immunosorbent Assay (ELISA) for the detection of SD on herd level (La et al., 2009; Song et al., 2012; Song et al., 2015).

In necropsy, a diffuse fibrinous to mucohaemorrhagic enteritis with pseudomembrane is found located in the large intestine. Histopathological lesions are mucosal oedema, invasion of lymphocytes and neutrophils, dilated colonic crypts filled with mucus and superficial erosion (Glock and Harris, 1972; Hampson et al., 2006). Especially in acutely infected animals, numerous spirochaetal bacteria are present. However, for a definitive diagnosis of SD, *B. hyodysenteriae* must be proved in the faeces or colonic mucosa. Though culture is effortful and time-consuming, it is the most practiced method for the detection of *B. hyodysenteriae*. Faeces, rectal swabs with medium and mucosal scrapings from necropsy are suitable samples. Cultures are incubated at 37°C to 42°C in anaerobic atmosphere for up to seven days. Traditionally, a selective agar based on trypticase soy agar (TSA) supplemented with various antimicrobials is used for culture, but since there is no recommended standard method, a variety of selective agars is applied. Initially, TSA and spectinomycin (TSA-S400) for the inhibition of the faecal concomitant flora was developed (Songer et al., 1976). Kunkle and Kinyon (1988) added pig faeces extract and colistin, spiramycin, rifampicin and vancomycin (BJ-agar). The BJ-agar was modified to BJ-Actidion agar by the addition of cycloheximide to inhibit mycological contamination (Dünser et al., 1997). Blood agar modified medium consisting of a blood agar base, defibrinated horse blood, beef extract and bacto peptone with spectinomycin and rifampin (BAM-SR) (Calderaro et al., 2001) in combination with a pre-treatment in brain heart infusion broth with spectinomycin and rifampin (Calderaro et al., 2005) supported growth of *B. hyodysenteriae* within 48 h. The addition of colistin, spectinomycin, rifampicin and flavomycin to blood agar modified medium (BAM-CSRF) enhanced the recovery rate of *B. hyodysenteriae*, but pre-enrichment enhanced the occurrence of contaminating faecal flora (Lugsomya et al., 2012). Microscopical examinations can give a hint for spirochaetal growth, but further characterisation is required. Phenotypical tests based on biochemical features (Fellström and Gunnarsson., 1995) and serological detection of antigens were widely used before molecular methods became more accessible. By now, both methods are widely replaced by polymerase chain reaction (PCR) (Elder et al., 1994). Common target genes are the 23S rDNA, *nox* gene, *tyl*A gene, (Leser et al., 1997; Atyeo et al., 1999; Fellström et al., 2001) and 16S rDNA for *B. pilosicoli* (Park et al., 1995; Fellström et al., 1997; Atyeo et al., 1998). Duplex PCRs detecting both swine pathogens *B. hyodysenteriae* and *B. pilosicoli* are described as well (La et al., 2003).

However, a PCR targeting *tyl*A gene for *B. hyodysenteriae* and 16S rRNA gene for B. *pilosicoli* was less sensitive for *B. hyodysenteriae* than culture in combination with biochemical typing (Råsbäck et al., 2006). For the simultaneous detection of different pathogens like *B. hyodysenteriae*, *Lawsonia intracellularis* and *Salmonella* spp., multiplex PCRs are established (Suh and Song, 2005). Effectiveness of PCR could even be enhanced by establishing real-time PCR (Akase et al., 2009). An alternative method for further analysis of cultured Brachyspira is MALDI TOF (Calederaro et al., 2013; Prohaska et al., 2014), but mixed culture of different *Brachyspira* spp. cannot be unambiguously identified, which is the main disadvantage compared to PCR.

2.7 Treatment

Though certain immunity is seen in convalescent pigs, a commercial vaccination is not available, and disease control focusses on biosecurity and antimicrobial treatment. Biosecurity measurements include disinfection, limited access to production site, quarantine control of rodents and insects as well as fencing off wild boars. All-in-all-out production systems are recommended, and new pigs should be purchased from SD-free farms. However, in case of disease, the only therapy option is antimicrobial treatment. Unfortunately, there is only a limited selection of antimicrobial agents available for the treatment of SD. Drugs are applied in food or drinking water, but in severe cases, animals should be treated parenterally since water and food intake are reduced in sick animals. Mortality of SD mostly occurs due to dehydration. There are two treatment strategies. In herds with persisting SD, therapeutic success is defined by the reduction or elimination of clinical signs without stopping the colonisation by *B. hyodysenteriae*. A comparable effect may be achieved by good management. However, eradication always is the preferred option. Hereby, the elimination of the agent is the main objective of therapy, and additional measures like depopulation, vector control and disinfection of manure are incorporated (Neto, 2008; Speiser et al., 2011). Despite the central role of antimicrobial treatment in SD control, antimicrobial susceptibility testing (AST) is not standardised. In general, mainly two different methods for AST of Brachyspira are performed: agar dilution and broth dilution. All tests determine the minimal inhibitory concentration (MIC) for the antimicrobial agents. The MIC is defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism (CLSI, Document VET01-A4, 2013). Agar dilution is the recommended method of AST of anaerobic bacteria (CLSI, Document M11-A8, 2012). Different concentrations of

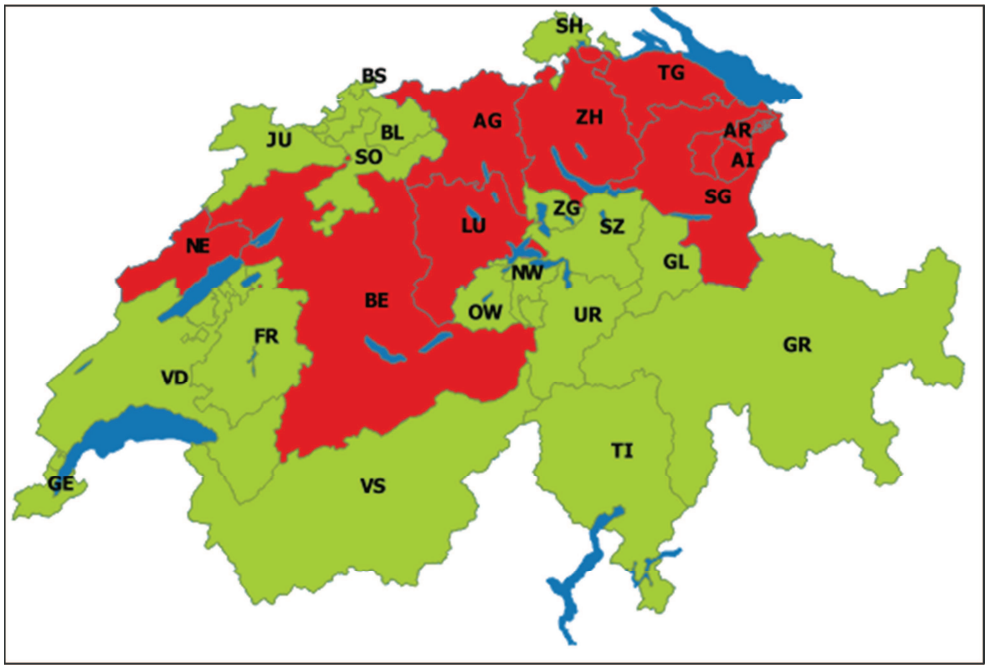
antimicrobials are moulded in agar plates and examined for bacterial growth. However, the difficulty of testing Brachyspira with agar dilution is the detection of visible growth. Brachyspira are slow-growing bacteria and initial growth can only be detected by the appearance of haemolysis. However, subinhibitory concentrations of antimicrobial agents may prevent haemolysis (Shibl and Gemell, 1983). Besides, agar plates are produced manually, which is laborious and susceptible to faults, especially if low concentrations are required. For the broth dilution, liquid medium with decreasing concentrations of an antimicrobial agent is used instead of agar plates. Visible growth is defined by the appearance of turbidity. It can be differentiated between macro- and microdilution based on the size of the volume. Microdilution tests are usually performed in microtitre plates encoated with the antimicrobial agents. For Brachyspira, a commercial microdilution test is available.

3 Materials and Methods

3.1 Isolates

For the antimicrobial susceptibility testing, 30 *B. hyodysenteriae* porcine field isolates were tested. They were cultured during routine diagnostics from 2009 to 2015 from nine different Swiss cantons (Fig. 1). For isolation, faecal swabs from pigs were streaked on a selective agar (BSA), a modified BJ-Actidion agar without pig faeces extract and cyclohexidime (Dünser et al., 1997). Agar plates were checked for spirochaetal growth by native microscopy after incubation at 42°C in an anaerobic atmosphere of 80% N_2, 10% CO_2 and 10% O_2 (Trilab, bioMérieux, Switzerland) for two to seven days. The temperature of 42°C is adjusted to inhibit concomitant intestinal flora. If samples were positive in native microscopy, they were subcultured on Columbia blood agar (Oxoid, Switzerland) for further molecular investigations. The PCR used in the diagnostic workflow is a duplex PCR for *B. hyodysenteriae* and *B. pilosicoli* (La et al., 2003) based on *nox* and 16S rRNA gene, respectively. Isolates were frozen in brain heart infusion (BHI) (Becton, Dickinson and Company, Switzerland) with glycerine (Sigma Aldrich, Switzerland) and foetal calve serum (Sigma Aldrich, Switzerland) at –80°C. Isolates were refrozen on Columbia blood agar (Oxoid, Switzerland) and checked by native microscopy for contaminations. Because of their swarming behaviour, cultures with more than one *Brachyspira* spp. are difficult to detect. In order to avoid mixed *Brachyspira* cultures, all isolates were tested for their purity by a real-time PCR (Borgström et al., 2016) based on 23S rRNA gene. Finally, isolates could be used for further testing.

Figure 1: Map of Switzerland. *B. hyodysenteriae* isolates investigated in the study are sampled from the red-coloured cantons.



3.2 Inoculum

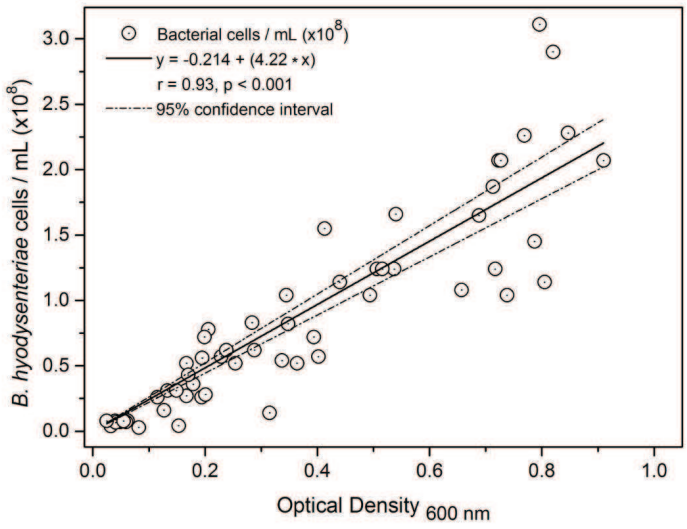
In order to obtain a precise inoculum for testing, cell counts of *B. hyodysenteriae* were set in proportion to the OD_{600} and a regression curve was developed. Therefore, *B. hyodysenteriae* ATCC reference strain was streaked on Columbia blood agar and incubated for three days at 42°C and then suspended in 2ml NaCl 0.9%. A serial dilution of the suspension was done (1:2, 1:4, 1:8 and 1:16). The optical density of all dilutions was measured at a wavelength of 600nm (BioPhotometer, eppendorf, Switzerland). Two times, 2 µl of the 1:8 dilution were transferred to an object slide, and ten visual fields of each were counted in a counting chamber at 400x magnification. The mean value was determined and was divided by 10 to obtain the mean value of spirochaetes per reticule (X), which was inserted into the following formula:

X x 2592 x 1000 x 8 = number of spirochaetes per ml

The volume under the coverslip is 2 µl, the area of the reticule fits 5184 times in the area of the coverslip. Therefore, the volume under the reticule is 0.000385802 µl. Hence, the number of spirochaetes has to be multiplied by 2592 to get the number of spirochaetes per µl and by

1000 to get the number of spirochaetes per ml. In order to take the dilution into account, a multiplication by 8 has to be done. Finally, the number of spirochaetes per ml is obtained. The optical density and the number of spirochaetes were set in proportion and a regression curve was created (Fig. 2).

Figure 2: The regression curve shows the correlation between bacterial cell count (bacteria/ml) and the OD_{600}.

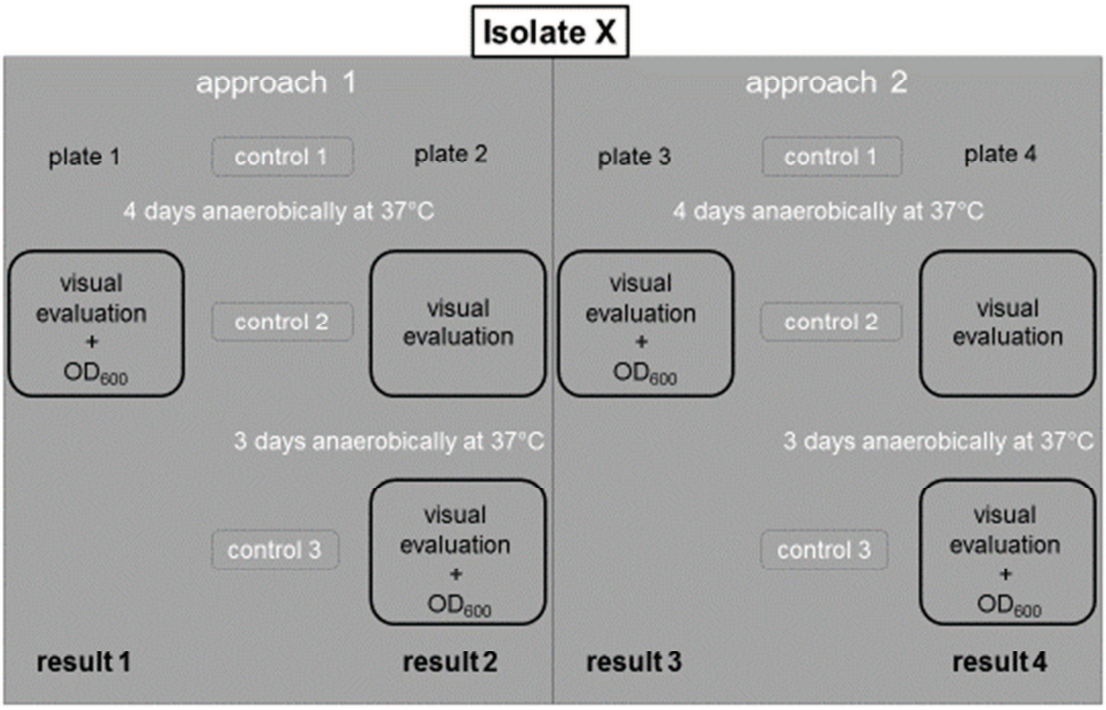


3.3 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed with the VetMIC Brachy panel (Statens veterinärmedicinska anstalt, Sweden), a commercial broth microdilution test. The antimicrobial agents included in the test are the pleuromutilins tiamulin (0.063 µg/ml to 8 µg/ml) and valnemulin (0.031 µg/ml to 4 µg/ml), the tetracycline doxycycline (0.125 µg/ml to 16 µg/ml), lincomycin (0.5 µg/ml to 64 µg/ml) as well as the macrolide tylosin (2 µg/ml to 128 µg/ml) and its derivate tylvalosin/aivlosin (0.25 µg/ml to 32 µg/ml). The VetMIC Brachy panel consists of a 48-well plate coated with the six different antimicrobials in arithmetically decreasing concentrations. The inoculation of the VetMIC Brachy panel was performed with modifications to the manufacturer's instructions and is described as follows: Isolates were streaked on Columbia blood agar and incubated at 42°C for three days. Cultures were harvested in brain heart infusion (Becton, Dickinson and Company, Switzerland) with 10% foetal calve serum (Sigma Aldrich, Switzerland) (BHI + FCS) until an optical density of 0.6 E to 0.8 E was reached. The suspension was diluted 1:1000 in BHI + FCS, corresponding to 2 to 3 x 10^7 bacterial cells per ml. For confirmation, a microscopical cell count of 10 visual fields

at 100x magnification was made. Two VetMIC plates in parallel were inoculated with 500 µl per well. An additional control plate without antibiotics as growth control and a negative control with BHI + FCS only was incubated. The three plates were incubated in an anaerobic jar (AnaeroGen 2.5 l, Thermo Scientific, Switzerland) at 37°C and shook initially for four days. In order to detect contamination, cultures were examined by native microscopy and were streaked on Columbia blood agar (aerobic and anaerobic at 37°C) three times (before the incubation of the VetMIC plates, at the first evaluation after four days and at the second evaluation after seven days). After four days, all plates were evaluated macroscopically with a mirror for turbidity. For the first plate, the OD_{600} of all turbid wells and at least two of the proximate clear wells was measured, while the second plate was incubated for another three days. After seven days, the second plate was again evaluated macroscopically, and this time the measurement of OD_{600} of all turbid wells and at least two of the proximate clear wells was performed as well (Fig. 3). Therefore, four MIC values were obtained for every isolate. ATCC strain 27164 was tested in parallel at every approach and every field isolate was tested twice.

Figure 3: The workflow of AST. Every isolate was tested four times in two approaches. Two plates per approach were tested under the same conditions and evaluated visually. For one plate, OD_{600} was additionally measured. The second plate was incubated and was evaluated after another three days of incubation. Controls were established before the testing, after four days and after seven days.



3.4 Sequencing

All tested isolates were sequenced for a point mutation in the 23S rRNA gene responsible for lincosamide and macrolide resistance as previously published (Karlsson, 1999). For DNA extraction, bacteria were harvested from Columbia blood agar and suspended in molecular grade water (Milli-Q, Millipore Corporation). After a centrifugation for 1 min at 12,000 rpm, the supernatant was rejected and the remaining pellet was resuspended in 200 µl lysis buffer (Instagen Matrix, Bio-Rad). Samples were incubated and shaken at 56°C for 15–30 min and afterwards for 8 min at 99°C. After another centrifugation at 12,000 rpm for 3 min, 100 µl of the supernatant were used. DNA concentrations were determined spectrophotometrically (NANODROP 2000c, Thermo Scientific). The primers used for the PCR are previously described by Karlsson et al. (1999). The primer concentration was 20 uM and the DNA template used 20 ng/µl. The master mix used and the PCR cycle conditions are presented in Figure 4. The expected fragment of 388bp was presented by capillary electrophoresis (QIAxcel, QIAGEN, Switzerland). The DNA was purified with the QIAquick PCR Purification Kit (QIAGEN, Switzerland) in QIAcube (QIAGEN, Switzerland) according to the manufacturer's protocol. The sequencing was performed externally by Microsynth AG (Switzerland). For the analysis of the obtained sequences, ApE (A Plasmid Editor) software was used.

Figure 4: Master Mix and PCR cycle conditions used for the sequencing of the 23S rRNA gene.

Components	µl per well
Fermentas Pfu DNA Polymerase (1.25u)	0.5
Primer forward (20uM)	1.25
Primer reverse (20uM)	1.25
PFU Buffer with MgSO4	5
Water	33
dNTPs	5
DNA	4
Volume total	50

PCR cycle conditions	
95°C 2min	
denaturation at 95°C for 30 s	40x
annealing at 55°C for 30s	
longation at 72°C for 1 min 30s	
72°C 10 min	

3.5. Antimicrobial Activity

The influence of the used medium (BHI + FCS), the atmosphere as well as the time of incubation on the activity of the antimicrobial agents was examined. Therefore, stock solutions of the six antimicrobials used in the testing were prepared. A concentration of 5.12 mg/ml was set for the stock solution to facilitate the following dilutions. Taking the potency of the agents into account, 51.3 mg tiamulin (Tiamulin fumarate VETRANAL), 52.8 mg valnemulin (Valnemulin-hydrochloride VETRANAL), 52.7 mg doxycycline (Doxycycline hyclate VETRANAL), 53.2 mg lincomycin (Lincomycin hydrochloride monohydrate VETRANAL) and 55.3 mg tylosin (Tylosin tartrate VETRANAL), respectively, were dissolved in 10 ml distilled water. The five aforementioned antimicrobial agents were all obtained from Sigma-Aldrich, Switzerland. For tylvalosin, 51 mg (Aivlosin/tylvalosin tartrate, EcoAnimal Health, United Kingdom) were solved in 5 ml ethanol (99%) and refilled with 5 ml distilled water.

In order to evaluate if BHI + FCS influences the activity of the antimicrobial agents, it was compared with Müller Hinton (MH) broth (Oxoid, Switzerland), the standard medium for AST. Therefore, a microdilution with *Staphylococcus aureus* ATCC 29213, a reference strain for AST and susceptible to all tested antimicrobial agents, was performed. An inoculum of 0.5 McFarland of *Staphylococcus aureus* ATCC 29213 was produced, diluted 1:100, and 50 µl per well were distributed in a 96-well microtitre plate. The stock solution was diluted 1:10 and a serial dilution from 256 µg/ml to 0.5 µg/ml was performed. Finally, every well contained 100 µl with 5 x 10^4 bacteria. This was done with BHI + FCS and MH broth. The test was incubated at 37°C between 17 h and 20 h. In order to examine the influence of atmosphere, plates were incubated aerobically and anaerobically. Furthermore, *Staphylococcus aureus* ATCC 29213 was tested with a VetMIC Brachy plate with BHI + FCS. The obtained MIC values were compared to each other and controlled with published MIC values of *Staphylococcus aureus* ATCC 29213 (Tab. 1). The influence of incubation time was evaluated as follows: BHI + FCS and MH broth, respectively, were incubated with the antimicrobial agents with the concentration of 512 µg/ml at 37°C aerobically and anaerobically for seven days altogether. Every 24 h an aliquot was taken off and frozen at –20°C. The aliquots were used as basis for the aforementioned serial dilution and tested against *Staphylococcus aureus* ATCC 29213. If antimicrobial agents were inactivated during this time, the concentration would decrease in every well and growth of *Staphylococcus aureus* ATCC 29213 would be detected in more wells.

Table 1: Quality range of *Staphylococcus aureus* ATCC 29213. MIC values obtained in our study by microbroth and VetMIC Brachy panel are compared to published MIC values.

agent	MIC µg/ml (our study)		MIC µg/ml	reference	method
	microbroth	VetMIC Brachy			
tiamulin	0.5 – 1	2	1	Karlsson et al., 2003	microbroth
			2	Lobová et al., 2004	agar dilution
valnemulin	≤0.25	0.25	0.125	Lobová et al., 2004	agar dilution
doxycycline	≤0.25	0.5			
lincomycin	≤0.25 – 0.5	2			
tylosin	1 – 2	2	≤2	Karlsson et al., 2003	microbroth
tylvalosin	2 – 4	4			

4 Results

4.1 Isolates

The study included 30 Swiss *B. hyodysenteriae* isolates collected from 2009 to 2015. For an accurate AST, the purity of isolates must be ensured. However, the field isolates thawed from the collection had a high rate of contamination. Native microscopy as exclusive investigation to detect contamination was insufficient, and additional controls in form of aerobic and anaerobic cultures on Columbia blood agar were established. Besides contamination with non-spirochaetal bacteria, mixed cultures with different *Brachyspira* spp. were quite common and were detected by a real-time PCR (Borgström et al., 2016) to be excluded from the study. Since subcultivation of the impure isolates was time-consuming, contamination aggravated the AST enormously and was the main criterion for the disqualification from the study.

4.2 Inoculum

One object of the study was the optimisation and evaluation of the broth microdilution for routine diagnostics. First of all, a method to establish a precise inoculum based on cell counting rather than colony-forming units was developed. Therefore, a regression curve, which shows the correlation between optical density and bacterial cell counting, was developed (Fig. 2). Optical density and bacterial cell counts showed a good correlation, and an inoculum of 2 to 3 x 10^7 could be adjusted.

4.3 Antimicrobial Activity

Additionally, BHI + FCS was compared to MH broth, the standard medium for AST. When tested against a susceptible *Staphylococcus aureus* ATCC 29213, MICs obtained from the attempts with BHI + FCS were in the quality range of *Staphylococcus aureus* ATCC 29213 (Tab. 1), and attempts with BHI + FCS did not differ more than one twofold dilution. Neither did the incubation at an anaerobic atmosphere influence the results of the AST of *Staphylococcus aureus* ATCC 29213. The MICs were the same when testing was performed under aerobic conditions. Besides, an inactivation of the antimicrobial agents over the time of seven days could not be observed.

4.4 Antimicrobial Susceptibility Testing

4.4.1 Determination of Minimal Inhibitory Concentrations

The antimicrobial susceptibility for Swiss *B. hyodysenteriae* isolates was determined and the MIC values which were obtained after four days of incubation are depicted in Figure 5. The MIC_{50} and MIC_{90} were calculated and are presented in Table 2. Visually detectable growth could be confirmed by OD_{600}.

The best susceptibility and a unimodal distribution of the isolates could be seen for the pleuromutilins (Fig. 5A/B). All isolates were inhibited at very low concentrations. Tiamulin had an MIC_{50} of ≤0.063 µg/ml and an MIC_{90} of 0.125 µg/ml. Both values were consistent in all four attempts. The MIC_{50} of valnemulin in the four attempts was ≤0.031 µg/ml. Differences of MIC_{90} could be seen between approach 1 and 2. The MIC_{90} for valnemulin in approach 1 was ≤0.031 µg/ml and 0.063 µg/ml in approach 2. For doxycycline, growth could be detected up to a concentration of 4 µg/ml with more isolates being inhibited at the lowest concentrations tested. The MIC_{50} of doxycycline was 0.25 µg/ml and MIC_{90} 2 µg/ml (three out of four attempts) and 1 µg/ml in one of the attempts. The AST of lincomycin parted isolates into two populations. Between 30% and 37% of the isolates were inhibited at ≤0.5 µg/ml. Three out of four attempts revealed an MIC_{50} of 16 µg/ml, whereas MIC_{50} was 8 µg/ml in one attempt. In all testings an MIC_{90} of 32 µg/ml could be determined. The highest MIC values were detected for tylosin. The highest concentration tested (>128 µg/ml) could not inhibit growth for 50% to 60% of the isolates. Therefore, MIC_{50} and MIC_{90} of tylosin were identical at >128 µg/ml. Isolates with high MIC values also showed increased MIC values for lincomycin. The testing of tylvalosin revealed an MIC_{50} of 4 µg/ml and 2 µg/ml, respectively, and MIC_{90} was 8 µg/ml.

Figure 5: Distribution of MICs (in µg/ml) obtained from all plates after four days of incubation. The percentage of isolates showing skips are presented on the right-hand side, since an accurate MIC determination is not possible if skips occur.

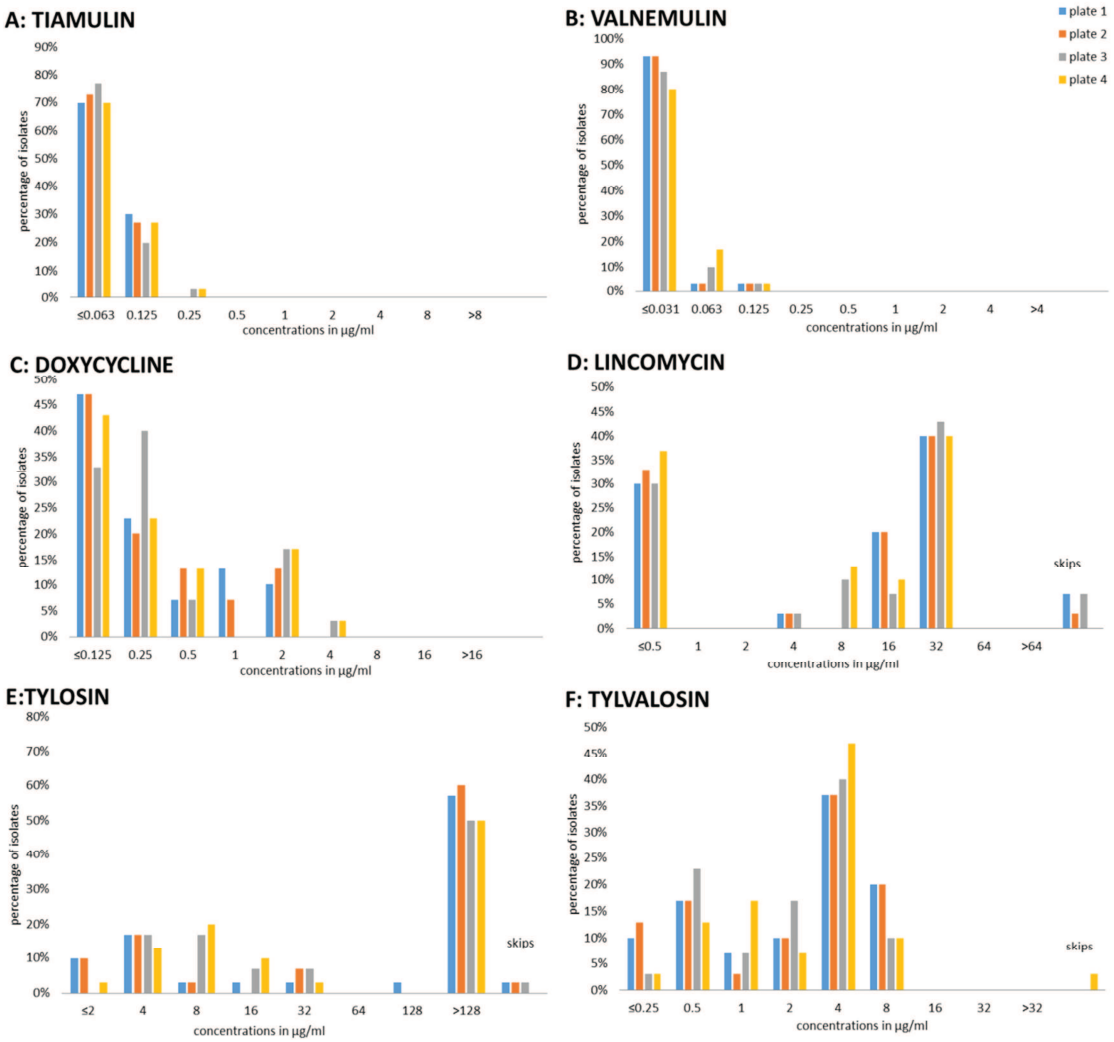


Table 2: MIC_{50} and MIC_{90} of all approaches after four and seven days, respectively.

	4 days								7 days			
antimicrobial agents	MIC50 in µg/ml				MIC90 in µg/ml				MIC50 in µg/ml		MIC90 in µg/ml	
	approach 1 plate		approach 2 plate		approach 1 plate		approach 2 plate		approach 1 plate	approach 2 plate	approach 1 plate	approach 2 plate
	1	2	3	4	1	2	3	4	2	4	2	4
tiamulin	≤0.063	≤0.063	≤0.063	≤0.063	0.125	0.125	0.125	0.125	≤0.063	≤0.063	0.125	0.125
valnemulin	≤0.031	≤0.031	≤0.031	≤0.031	≤0.031	≤0.031	0.063	0.063	≤0.031	≤0.031	≤0.031	0.063
doxycycline	0.25	0.25	0.25	0.25	1	2	2	2	0.25	0.25	2	4
lincomycin	16	16	16	8	32	32	32	32	32	32	32	/*
tylosin	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
tylvalosin	4	4	2	4	8	8	8	8	4	4	16	32

* MIC_{90} could not be determined after seven days in approach 2, since 17% of the isolates showed skips.

4.4.2 Reproducibility and Sequencing

For evaluation of the reproducibility, every isolate was tested four times. The comparison of the four MICs obtained for the same isolates after four days of incubation revealed the following results: For the pleuromutilins and doxycycline the test showed a good reproducibility. Within an approach under the same conditions performed on the same day with the same inoculum, MIC values never differ more than onefold doubling dilution (Fig. 6A). The same could be applied for the pleuromutilins when the two approaches performed on different days were compared (Fig. 6B). For doxycycline, 23% of the isolates differed in MIC values two doubling dilution steps between the two approaches (Fig. 6B). Similar results were obtained by the parallel testing of *B. hyodysenteriae* ATCC 27164 for the pleuromutilins and doxycycline (Tab. 3). No deviation in MICs was observed for tiamulin. The range for valnemulin was ≤0.031 µg/ml to 0.063 µg/ml and for doxycycline ≤0.125 µg/ml to 0.5 µg/ml. However, for lincomycin and the macrolides, between 3% and 10% of the isolates within the same approach and 3% to 17% between the two approaches had MIC values differing more than two doubling dilutions (Fig. 6A/B). Besides, the control strain, which is originally inhibited at low concentrations of lincomycin and macrolides, grew in some approaches up to concentrations of 32 µg/ml (lincomycin), >128 µg/ml (tylosin) and 8 µg/ml (tylvalosin), respectively (Tab. 3). The deviation of the isolates based on so-called skips, a phenomenon where growth is already inhibited but can again be detected in wells with higher antimicrobial concentrations (Fig. 7). The percentage of isolates showing skips are displayed in Figure 5. Skips only occurred for lincomycin and the macrolides and influenced the reproducibility of testing. Since mutation is a possible origin for skips, isolates were sequenced for point mutations in the 23S rRNA gene. For every isolate the 388bp fragment could be detected by PCR (Fig. 8A) Isolates with high lincomycin and macrolide MIC values had an A -> T conversion (Fig. 8B; Tab. 4). The lowest concentration observed in mutated isolates was 4 µg/ml for lincomycin, 8 µg/ml for tylosin and 1 µg/ml for tylvalosin. Skips could only be detected in wild-type isolates except for two mutated isolates (No. 23 and No. 684), which showed skips for tylvalosin (Tab. 4). In a further step, *B. hyodysenteriae* was subcultivated out of the isolated turbid wells with the higher antimicrobial concentration and sequenced for the mutation. Sequences from subcultivations from skipped wells revealed an A -> G conversion. The same conversion could be detected for the control strain with the increased MIC values for lincomycin and tylosin, where growth was detected up to a

concentration of >128 µg/ml for tylosin. Sequences from subcultivations out of wells with concentration of 8 µg/ml tylosin were either wild-type or mutated.

Figure 6: Variance of MIC values after four days of incubation. Fig. 6A presents the variance within an approach and Fig. 6B the variance between approaches 1 and 2 performed on two different days. The red colour highlights the isolates which had the same MIC value in the different testings. The grey colour represents the deviation of one doubling dilution and the blue colour the deviation of two doubling dilutions. The green colour presents isolates differing more than two doubling dilution steps including isolates where skips occur.

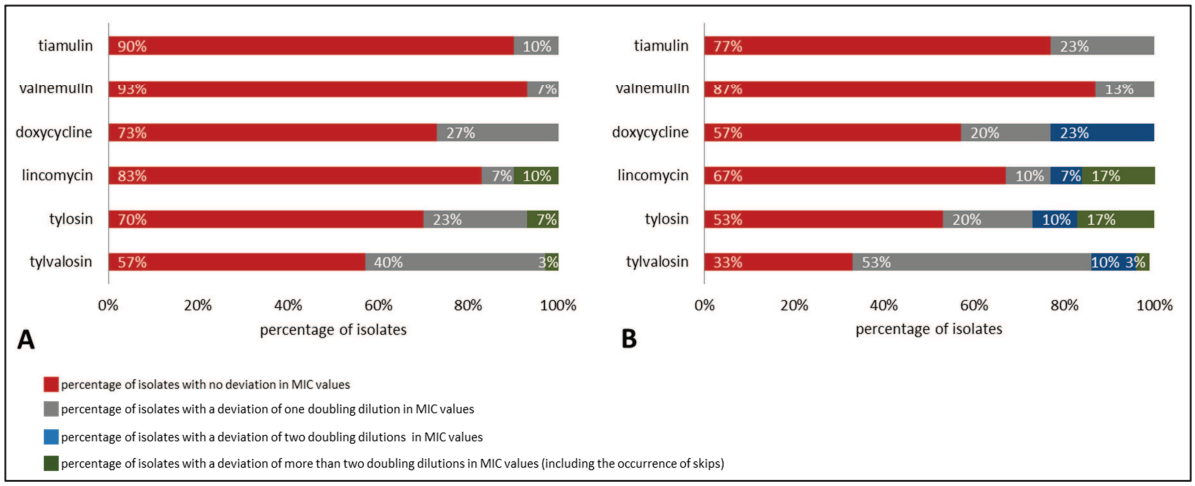


Table 3: Range of MIC values for *B. hyodysenteriae* ATCC 27164. The range of MIC values for *B. hyodysenteriae* ATCC 27164 obtained in our study is compared with the range of MIC values obtained in other studies. It is kept in mind that different testing methods influence the results.

agent	MIC µg/ml (our study)	MIC µg/ml	reference	method
tiamulin	≤0.063	≤0.031 – 0.062	Lobová et al., 2004	agar dilution
		0.03 – 0.06	Karlsson et al., 2003	microdilution
		0.031	Karlsson et al., 2002	broth dilution
		0.063	Karlsson et al., 2001	broth dilution
		0.0125 – 0.025	Kitai et al., 1987	agar dilution different inocula
		0.1	Rønne, Szancer, 1990	agar dilution
		0.08	Messier et al., 1990	agar dilution
		0.016 – 1	Pringle et al., 2006	VetMIC Brachy
valnemulin	≤0.031 – 0.063	≤0.031	Lobová et al., 2004	agar dilution
		0.063	Karlsson et al., 2001	broth dilution
		0.008 – 0.25	Pringle et al., 2006	VetMIC Brachy
doxycycline	≤0.125 – 0.5	0.063 – 0.5	Pringle et al., 2006	VetMIC Brachy
lincomycin	≤0.5 – 32	≤ 1	Karlsson et al., 2002	broth dilution
		10	Messier et al., 1990	agar dilution
		0.125 – 1	Pringle et al., 2006	VetMIC Brachy
tylosin	≤2 – >128	4 – 8	Karlsson et al., 2003	microdilution
		4	Karlsson et al., 2002	broth dilution
		1.56 – 12.5	Kitai et al., 1987	agar dilution different inocula
		14	Rønne, Szancer, 1990	agar dilution
		1 – 16	Pringle et al., 2006	VetMIC Brachy
tylvalosin	≤0.25 – 8	0.5 – 4	Pringle et al., 2006	VetMIC Brachy

Figure 7: Photography of a VetMIC Brachy plate with skipped wells for lincomycin and tylosin. The red-encircled wells show growth although growth is inhibited in the three previous wells with lower concentration of antimicrobial agent. Antimicrobial agents top down are: tiamulin, valnemulin, doxycycline, tylvalosin, lincomycin, and tylosin.

Figure 8: Results of the sequencing of the 23S rRNA gene. Fig. 8A shows the 388bp fragment of the 23S rRNA gene for 12 out of the 30 field isolates. An A -> T conversion could be detected in the sequence presented in Fig. 8B (red circle).

Table 4: Range of MIC values for lincomycin, tylosin and tylvalosin. Wild-type isolates (=wt) are highlighted in grey. Isolates with mutation showed an A -> T conversion.

isolate	lincomycin (MIC in µg/ml)	tylosin (MIC in µg/ml)	tylvalosin (MIC in µg/ml)	point mutation
21	32	>128	4 – 8	A → T
23	32 – 64	>128	4 – 16 (skips)	A → T
25	≤0.5 (skips)	8 – >128 (skips)	0.5 – 2	wt
30	32 – 64	>128	8 – 32	A → T
42	≤0.5	4 – 8	0.5	wt
97	≤0.5	<2 – 8	<0.25 – 1	wt
112	≤0.5 (skips)	<2 – 8 (skips)	<0.25 – 2	wt
153	32	>128	4 – 16	A → T
156	8 – 32	>128	2 – 8	A → T
158	4 – 32	128 – >128	2 – 4	A → T
231	≤0.5 (skips)	4 – 64 (skips)	0.5 – 1 (skips)	wt
257	≤0.5 (skips)	4 – 8 (skips)	0.5 – 1	wt
333	16 – 32	>128	4 – 32	A → T
340	32	>128	4 – 32	A → T
342	4 – 32	>128	1 – 4	A → T
344	16 – 32	>128	2 – 8	A → T
455	32	16 – 32	4	A → T
493	≤0.5	≤2 – 4	≤0.25 – 0.5	wt
526	≤0.5	32	2 – 4	wt
630	32	>128	4 – 8	A → T
639	≤0.5 – 1	4 – 8	0.5 – 1	wt
660	16 – 32	>128	4 – 8	A → T
684	32	>128	4 – 16 (skips)	A → T
696	32 – 64	>128	8 – 16	A → T
722	≤0.5	≤2 – 16	≤0.25 – 1	wt
753	≤0.5 (skips)	≤2 – 8	≤0.25 – 0.5	wt
783	16 – 32	8 – >128	4 – 32	A → T
784	8 – 32	>128	2 – 8	A → T
819	32	16 – >128	4 – 8	A → T
480	16 – 32	8 – >128	4 – 8	A → T

4.5 Incubation Time

The evaluation after seven days revealed in 53 out of 60 approaches an increase of MIC values for at least one antimicrobial agent (Fig. 9). If an increase of MIC values occurred in the first approach, it was not inevitably seen in the second approach of the same isolate. An increase could be observed for all antimicrobial agents (Fig. 9). The deviation was the least for valnemulin, where 97% (approach 1) and 90% (approach 2) of the isolates had the same MIC value at day four and day seven (Fig. 10). The highest increase was seen for tylvalosin, where only 33% (approach 1) and 40% (approach 2) of the isolates did not differ in their MIC values after the three days of additional incubation. A shift towards higher MIC_{50} and MIC_{90} was seen for doxycycline, lincomycin and tylvalosin (Tab. 2). However, time did not influence the antimicrobial activity of the agents, when tested against the susceptible *Staphylococcus aureus* ATCC 29213.

Figure 9.1: Evaluation of three days additional incubation of approach 1. Blue bars show the MIC values of the isolates after four days of incubation and orange bars the ones after seven days of incubation. A shift towards higher MIC values could be detected after seven days of incubation. The occurrence of skips is depicted at the right.

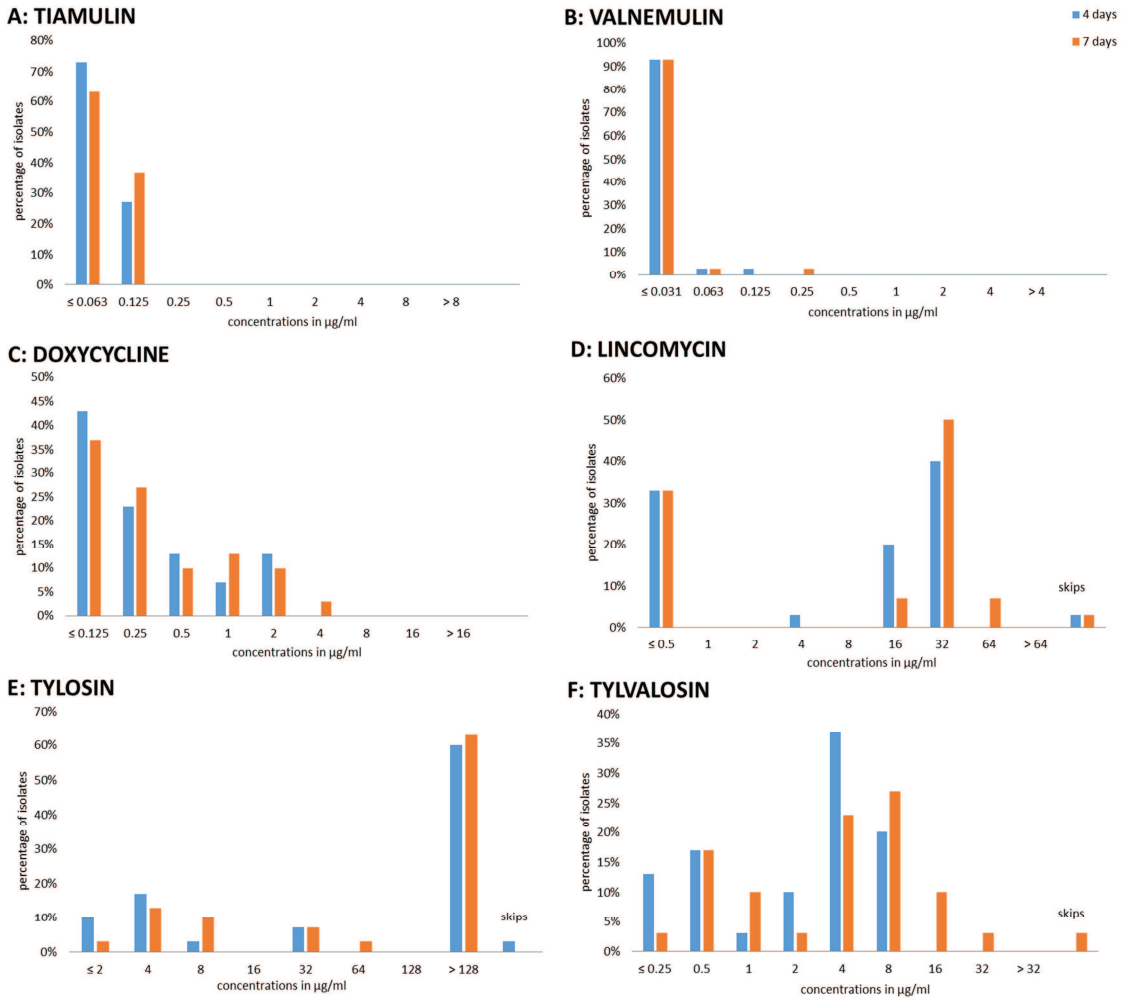


Figure 9.2: Evaluation of three days additional incubation of approach 2.

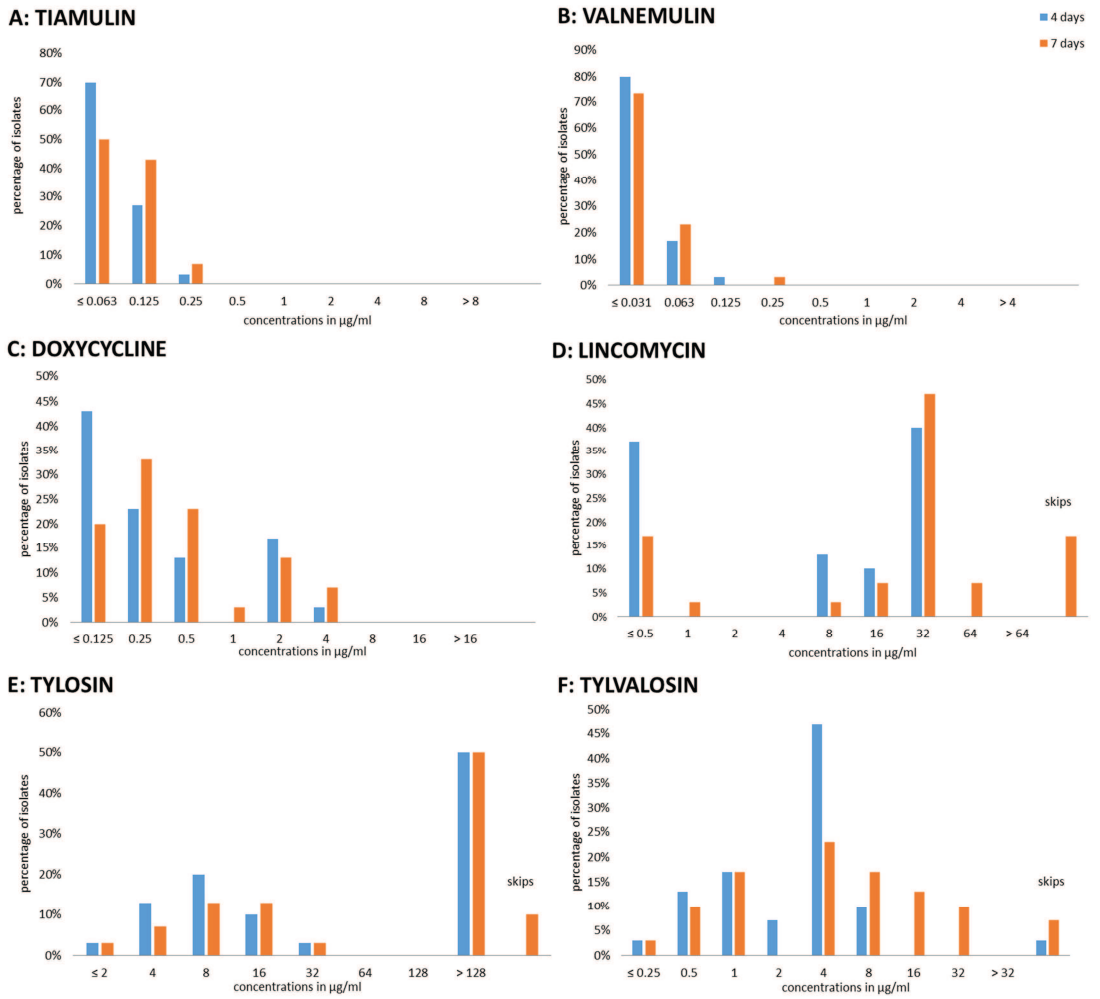
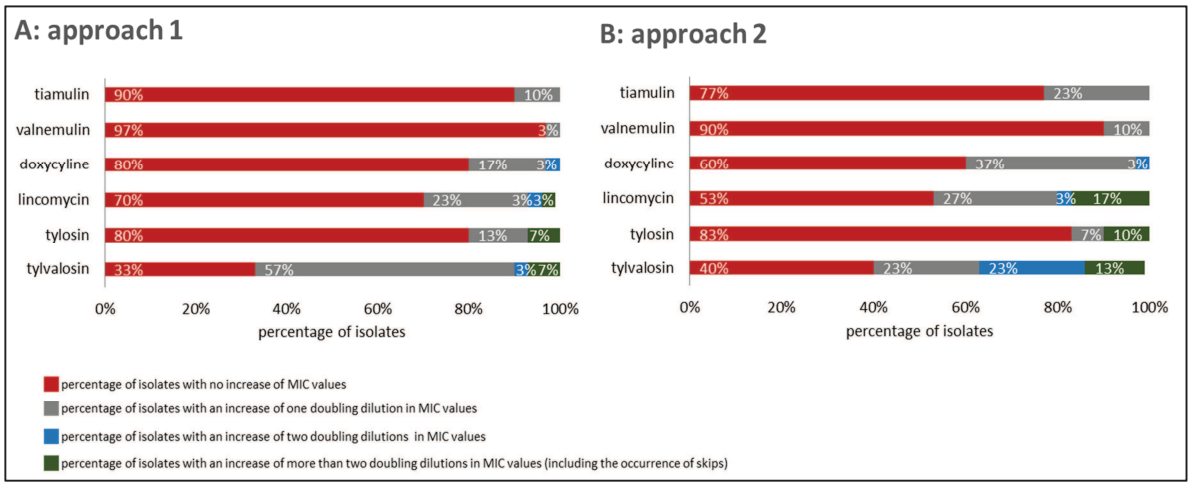


Figure 10: Increase of the MIC values after three days additional incubation. Approach 1 is presented in Fig. 10A and approach 2 in Fig. 10B. The red colour highlights isolates which showed no increase of MIC values after seven days. Grey-coloured isolates showed an increase of the MIC values of one doubling dilution and blue two doubling dilutions. The green colour presents isolates with an increase of more than two doubling dilutions including isolates where skips occurred.



5 Discussion

5.1 Isolates

The selection of isolates comprises a collection of samples from different farms located in nine Swiss cantons from 2009 to 2015. Concerning the period of time, the collection is representative for Switzerland because *B. hyodysenteriae* was first detected in 2008. However, in a geographical context, only parts of Switzerland are covered. Most of the isolates are obtained from the surveillance programme performed for the Swiss Pig Health Service. The membership in the Swiss Pig Health Service is voluntary, but participating farms are investigated for *B. hyodysenteriae* and *B. pilosicoli* twice a year. Therefore, the selection of isolates reflects where SD is investigated regularly. For unknown reasons, high contamination rates of the thawed isolates were observed. The swarming of *B. hyodysenteriae* aggravates the visual detection of contamination, and for further control native and darkfield microscopy, respectively, are widely used. In our study the sole use of native microscopy was not sufficient and supplementary controls had to be established. High contamination rates (42%) were also observed in a Swedish study with isolates from birds (Jansson et al., 2011). The authors assume that the contaminating bacteria adapt faster to the changing conditions after thawing and then overgrow the slow growing Brachyspira. Microscopical controls of culture before freezing is recommended. Filtration, which was used in the Swedish study to eliminate the contamination, was not successful in our study. Besides, the involvement of other anaerobic bacteria (Meyer et al., 1974; Whipp et al., 1979) in the pathogenesis of SD was presumed initially. Even though their role in pathogenesis could not be confirmed, they are able to aggravate clinical outcome. Co-infection may lead to co-culturing and finally to contamination. Bacterial interactions *in vitro* are only investigated for bacteria inhibiting the growth of *B. hyodysenteriae* (Bernadeau et al., 2009). Synergistic effects in culture have not been examined. Furthermore, antimicrobial resistance of *Enterobacteriaceae* is progressive, and the antimicrobials used in the selective agar for inhibition of concomitant flora may be ineffective (Lugsomya et al., 2012). The contaminating bacteria in our study were not further identified. The optimisation of culture is not in the focus of research anymore, and effective methods to clone Brachyspira is not yet developed, which aggravates accurate AST.

5.2 Inoculum

Besides the purity of isolates, a precise inoculum is essential for AST and it can be determined with the help of two methods. The first one is based on viable cell counts in form of colony-forming units (CFU), which is recommended by the manufacturer of the VetMIC Brachy panel. The decision for CFUs was made because *B. hyodysenteriae* coil under suboptimal conditions and bacterial cell counting may include cells which are not viable anymore (Karlsson et al., 2003). However, *B. hyodysenteriae* does not form colonies under normal conditions, and methods inhibiting the swarming on agar plates may as well have inhibiting effects on growth. Therefore, we decided to use an inoculum based on bacterial cell counts. In order to avoid the counting of dead cells, only bacteria with the typical spirochaetal form were included. The development of the regression curve allowed a conclusion from the OD_{600} to the number of bacteria. Nevertheless, the measurement of OD is laborious and time-consuming and is not a suitable method for routine diagnostics. The influence of different inoculum sizes was not determined in our study. For agar dilution, an influence of inoculum size on MIC values of four reference strains was observed depending on the antimicrobial agent tested (Kitai et al., 1987). For the microdilution test, this could not be confirmed (Karlsson et al., 2003).

5.3 Antimicrobial Activity

The comparison with MH broth revealed BHI + FCS to be a suitable medium for the AST of *B. hyodysenteriae*. Deviations of the standard medium for fastidious bacteria are allowed, but the suitability of the medium must be proved (CLSI, Document M11-A8, 2012). The time of incubation did not influence the MIC values of *Staphylococcus aureus* ATCC 29213. However, an increase of MIC values after three days additional incubation could be seen for the *B. hyodysenteriae* field isolates, especially for tylvalosin. Since inactivation of the antimicrobial agents could be excluded, the reason for the increase is not known.

5.4 Antimicrobial Susceptibility Testing

The data for antimicrobial susceptibility obtained for the tested Swiss isolates reflect an agreeable situation in Switzerland compared to other European countries. Progressive resistance is observed in many countries, which is alarming regarding the limited selection of antimicrobial agents licensed for the treatment of SD (Novotná and Škardová, 2002; Rohde

et al., 2004; Šperling et al., 2011, Hidalgo et al., 2011). The admitted antimicrobial agents in Switzerland include tiamulin, valnemulin and lincomycin. There is no product available containing tylvalosin. Products containing tylosin and doxycycline are licensed for swine, but the treatment of SD is off-label and requires rededication. However, for a proper rededication of drugs, data of antimicrobial susceptibility are essential. This leads to the next difficulty concerning the AST of *B. hyodysenteriae*. There are no standardised clinical breakpoints for the correct interpretation of results. Interpretation has to be carried out based on two publications, which supply divergent breakpoints for the classification. The first one (Rønne and Szancer, 1990) delivers breakpoints for tiamulin, lincomycin and tylosin. The breakpoint determination is based on pharmacokinetics in pigs based on the ratio between MIC values obtained with agar dilution and concentrations of antimicrobials in colonic tissue or serum (Tab. 5). The second publication takes colonic content concentrations, MIC values and, if available, clinical data into account and provides breakpoints for tiamulin, valnemulin, lincomycin, tylosin and tylvalosin (Burch, 2005). Besides, the second study differentiates between the testing methods used, since agar dilution, at least for the pleuromutilins, leads in average to MIC values which are one dilution step lower than microbroth does (Rohde et al., 2004). Furthermore, caution has to be taken with the interpretation of clinical studies because there are two therapeutic approaches. Therapeutic success can be defined as the reduction or elimination of clinical SD without stopping colonisation by *B. hyodysenteriae*, referring to herds which are persistently infected. However, the elimination of the agent is essential for eradication programmes and different treatment protocols are needed. Thus, interpretation of results becomes complex and ambiguous.

Table 5: Breakpoints for the classification. The breakpoints suggested by Rønne and Szancer (1990) are determined by agar dilution. The dosage of the antimicrobial agent is not specified. Isolates are classified in susceptible, intermediate and resistant for tiamulin, lincomycin and tylosin. Burch (2005) suggests breakpoints with the dosage of the antimicrobial agent for tiamulin, valnemulin, lincomycin, tylosin and tylvalosin determined by microbroth and agar dilution, respectively.

antimicrobial agent	MIC values in µg/ml					dosage (level in feed in ppm) Burch, 2005
	Rønne and Szancer, 1990			Burch, 2005		
	agar dilution			microbroth	agar dilution	
	susceptible	intermediate	resistant	resistant	resistant	
tiamulin	≤1	>1 to ≤4	>4	>0.5	>1.0	100
valnemulin	/	/	/	>0.125	>0.25	75
lincomycin	≤4	>4 to ≤36	>36	>50	>100	110
tylosin	≤1	>1 to ≤4	>4	>16	>32	100
tylvalosin	/	/	/	>16	>32	100

Rønne and Szancer (1990) categorize isolates ≤1 µg/ml as susceptible and >4 µg/ml as resistant to tiamulin. In a study including the clinical efficacy of therapy (Vyt and Hommez, 2006), the treatment with tiamulin was inefficient for isolates with MICs >1 µg/ml. This is in accordance with the investigation of 72 Swedish *B. hyodysenteriae* field isolates showing that the susceptible wild-type population had MIC values below 0.5 µg/ml (Karlsson et al., 2003). Thus, a lowering of the breakpoint to >0.5 µg/ml and >1 µg/ml, respectively, is postulated (Karlsson et al., 2003; Lobová et al., 2004; Vyt and Hommez, 2006). Since MIC values for pleuromutilins are in average one fold lower when tested in microbroth instead of agar dilution, Burch (2005) suggests a breakpoint of 0.5 µg/ml for isolates tested with microbroth and 1 µg/ml for isolates tested by agar dilution. All Swiss isolates tested in this study were susceptible to tiamulin irrespectively of the breakpoint used. The same applies to valnemulin with all isolates showing full susceptibility using the breakpoint of <0.125 µg/ml. High efficacy of pleuromutilins cannot be taken for granted since reduced susceptibility is quite common (Karlsson et al., 2002; Lobová et al., 2004; Molnàr, 1996; Rohde et al., 2004). In Spain, the treatment with pleuromutilins can be regarded as ineffective against one third of the isolates (Hidalgo et al., 2009). Furthermore, multiresistant isolates occur in Spain and Czech Republic (Šperling et al., 2011; Hidalgo et al., 2011). Progression of resistance can be presumed (Molnàr, 1996) and exacerbates the situation. In Sweden, a country comparable to Switzerland concerning the health status of pig herds and the use of antimicrobial drugs, a gradual increase of MIC values for tiamulin could be detected in the period between 1990 and 2003 (Pringle et al., 2012). However, the increase stagnated, which the authors lead back to a

national eradication programme with a controlled use of tiamulin. Besides, the stagnation is in good agreement with the slow and stepwise development of resistance described for the pleuromutilins (Karlsson et al., 2001; Karlsson et al., 2004a; Lobovà et al., 2004), which is an indication for the involvement of several genes. Though mutations of the L3 and 23S rRNA gene leading to alterations of the ribosomal binding site were found in isolates with reduced pleuromutilin susceptibility (Pringle et al., 2004; Hidalgo et al., 2011; Hillen et al., 2014), the resistance mechanism is not fully understood. The pleuromutilins showed the best reproducibility in the test. Both antimicrobial agents never differed more than one twofold dilution within an approach and between the two approaches after the four day evaluation. The increase after three days additional incubation did not go beyond one twofold dilution step either. That could be applied for the field isolates and the control strain ATCC 27164. Since tiamulin and valnemulin are licensed for the treatment of SD, the reliable results are quite important. However, all isolates had very low MICs, and whether good reproducibility is stable for isolates with higher MIC values can only be presumed. In an interlaboratory study, a multiply resistant *B. hyodysenteriae* isolate failed as potential quality control strain for antimicrobial susceptibility testing by broth dilution (Pringle et al., 2006).

For doxycycline, no breakpoints are available. In a genetic investigation of the bimodal population seen for German and Swedish field isolates, a point mutation of the 16S rRNA gene, correlating to a known tetracycline binding site in the 30S ribosomal subunit, could be detected (Pringle et al., 2007). Susceptible wild-type isolates had MICs from 0.125 µg/ml to 0.25 µg/ml and the mutated isolates had MICs between 1 µg/ml and 4 µg/ml. This is in good accordance with the findings in our study and using the cut-off value of ≥1 µg/ml (Zmudszki et al., 2012), 20% of the Swiss isolates showed reduced susceptibility to doxycycline. The reproducibility for doxycycline was in an acceptable range. No deviation greater than two twofold dilutions was observed.

Lincomycin is one of the admitted antimicrobial agents for Switzerland and therefore the antimicrobial susceptibility data are of special interest. About one third of the isolates could be inhibited at the lowest concentration (0.5 µg/ml). Then a gap with no isolates having the MICs of 1 µg/ml and 2 µg/ml occurs, and then growth could be detected up to concentrations of 32 µg/ml. Applying the breakpoint of ≤4 µg/ml (Rønne and Szancer, 1990), about one third of the isolates would be susceptible. The rest of the isolates could be comprised as intermediate (>4 µg/ml to ≤16 µg/ml). However, there are hints that *in vitro* testing is not in agreement with clinical outcome since therapy with lincomycin was successful in strains classified as resistant according to Rønne and Szancer (1990) (Smith, 1990; Vyt and

Hommez, 2006). Hence, Burch (2005) elevated the breakpoint to >50 µg/ml. Subsequently, lincomycin would be efficient against all tested isolates. The MIC values for tylosin of half of the isolates were higher than the range tested (>128 µg/ml). Consequently, 69% to 83% of the isolates are resistant according to Rønne and Szancer (1990). Some isolates cannot be categorized since the VetMIC Brachy panel does not include the breakpoint of ≤1 µg/ml for susceptible isolates. Between half and two thirds of the isolates are classified as resistant to tylosin applying the breakpoint of >16 µg/ml (Burch, 2005). The rest of the isolates could be categorized as susceptible. For tylvalosin, susceptibility of all isolates could be determined (Burch, 2005). The genetic base of lincomycin and tylosin resistance is well understood for *B. hyodysenteriae*. Because of the common binding site at the 50S ribosomal subunit, cross-resistance between macrolides, lincosamides and streptogramins (MLS_B-resistance) is usually observed (Vester and Douthwaite, 2001). In the case of *B. hyodysenteriae*, a point mutation of the 23S rDNA is described (Karlsson et al., 1999). The 30 isolates were sequenced for the occurrence of the point mutation. Wild-type isolates without a mutation in the 23S rDNA sequence showed great ranges for MICs and additionally skips in testing. Sequencing of populations isolated from separated turbid wells where skips occurred in testing could verify the existence of the mutation in original wild-type isolates. Wild-type isolates had MIC values up to concentrations of 4µg/ml, which correlates with the clinical breakpoints for intermediate isolates (Rønne and Szancer, 1990). Mutated isolates showed two different MIC distributions with one group having MICs >128 µg/ml and a few isolates with lower MICs from 8 to 32 µg/ml. Two strains had a range of 8 to >128 µg/ml and 16 to >128 µg/ml, respectively. An explanation could be that there is another resistance mechanism that parts the strains into high-level resistance isolates and low-level resistance isolates. From a clinical perspective this subdivision becomes relevant if the breakpoints of Burch (2005) are applied. Isolates with MICs of 8 µg/ml and 16 µg/ml would be classified as susceptible although they have an acquired resistance mechanism in form of a mutation. The same is seen for lincomycin, where isolates with point mutation are comprised in the susceptible range. It is possible that clinical outcome is still effective, but then the resistance mechanism responsible for clinical resistance is not known. Consequently, a phenotypic classification must be performed. However, the reproducibility of the test for lincomycin and macrolides was strongly influenced by the occurrence of skips leading to unreliable results. The occurrence of skipped wells has been previously described for *B. pilosicoli* (Karlsson et al., 2004b; Mirajkar and Gebhart, 2016) but not for *B. hyodysenteriae*, yet. Since contamination in skipped wells could be excluded and manufacturing error is unlikely, mutation or tolerance of the isolates are the remaining

explanations for skips. As cloning of *B. hyodysenteriae* is not yet possible, more than one clone may be present in the inoculum, and if the amount of mutated clones is sufficient, growth can be detected in higher concentrations. However, in purely statistical terms, one would expect a continuous elevation of MIC values rather than skips like it was seen in the control strain because the ratio of mutated and non-mutated clones should be the same in every single well. The second explanation is that mutation may take place in wells with higher concentrations during incubation. The sequencing does not fully clarify the question. Subcultures of wells with low concentrations showed the wild-type genotype. No bacterial growth was yielded from subcultivations of clear wells between two turbid wells. Subcultured bacteria of the skipped wells with the higher antimicrobial concentration were mutated. Whether a pure clone is present or one clone is predominant cannot be clarified by sequencing. Karlsson et al. (2004b) assume that the lower genetic stability of *B. pilosicoli* is responsible for the occurrence of skips and concludes that the microdilution test is less suitable for *B. pilosicoli*. However, susceptible *B. hyodysenteriae* strains became resistant *in vitro* after subculture on agar containing 4 µg/ml tylosin (Karlsson et al., 1999), and the reverse effect of a resistant strain becoming susceptible again could be observed as well (Kitai e al., 1987). Time and again, unusual phenomena are seen in AST of bacteria. The paradoxical effect defines the increase of surviving bacterial cells proportional to concentrations higher than the minimal bactericidal concentration (Eagle, 1948). It is best described for *Staphylococcus aureus* and β-lactam antimicrobials, but the paradox occurs for different bacterial species and antimicrobial classes (Amsterdam, 1996). In our case, skips were seen sporadically at different concentrations and were not reproducible. Hence, the occurrence of skips is not a paradoxical effect in the strict sense.

In conclusion, the evaluation of antimicrobial susceptibility of *B. hyodysenteriae* isolates from Switzerland was overdue, broth microdilution gave an overview of the Swiss situation, and trends towards lower susceptibility are detectable. However, the precise determination of MICs has to be treated with caution, and an accurate classification of isolates into susceptible and resistant isolates is challenging due to the lack of unambiguous pharmacological data. Furthermore, comparisons between studies are difficult since methods and interpretations are not uniform. AST of *B. hyodysenteriae* is still methodically immature and further standardisations must be established.

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